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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

STEWART, et al.

Serial No.: 09/438,944 Examiner: A. DeCloux

Filed: 11/12/1999 Group Art Unit: 1644

For: Compositions and Methods for Producing Vascular Occlusion

AFFIDAVIT UNDER RULE 1.132

The undersigned, Michael W. Stewart, hereby declares and says that:

1. I am an inventor in patent application Serial No. 09/438,944;

2. Of my personal knowledge and under my personal direction, the following experiments were performed as described:

Effects of Platelet-mediated vascular occlusion therapy on normal mice -

Pharmacokinetic and preliminary toxicological analysis of the platelet-mediated vascular occlusion therapy protocol on control SCID mice (i.e. non-tumor-bearing, n = 5) showed no macroscopic signs of non-specific thrombosis or other ill effects. Histological analysis of samples taken from the major organs showed no signs of microscopic thrombosis.

As part of the experimental protocol, human platelets were injected into the SCID mice since in vitro studies found that immobilized human VWF induced mouse platelet adherence only, while a mixture of mouse and human platelets resulted in dramatic upregulation of mouse and human platelet binding to VWF immobilized on polystyrene beads. The SCID mice (n = 5) tolerated the injection of human products (biotinylated human VWF, human platelets) well, with no external evidence of thrombosis or symptoms of distress. Human platelets were detected in mouse blood up to 2 hours post-injection, with complete disappearance of the platelets by 24 hours. Differentiation of human and mouse platelets was accomplished both morphologically (Coulter impedance sizing) and functionally (platelet activation by bead-immobilized human VWF). Mouse

Mice - platelets but patient immune VWF

platelets were noted to be significantly smaller in size and to weakly adhere to immobilized VWF. However, in the presence of human platelets, the mouse platelets synergistically reacted to form large thrombi (in vitro) with the human platelets and the VWF-coated beads.

Effects of Platelet-mediated Vascular Occlusion Therapy on Tumor-bearing mice.

SCID/beige mice were injected with LNCaP prostate cancer cells (10^6 cells, subcutaneous flank injection), developed palpable tumors in approximately 6 weeks (1 – 1.5 cm in diameter) and were randomized into two groups: 1) Treatment (11 animals; mean tumor diameter 1.2 ± 0.2 cm) and 2) Control (10 animals; mean tumor diameter 1.3 ± 0.2 cm). The mice were anaesthetized with a ketamine compound (IP injection) then sequentially injected (lateral tail vein) as follows:

Primary antibody (Humanized anti-MUC1.biotin or Control MOPC-21.biotin; 40 µg/ml, final),
Wait 10 minutes,
Avidin (10 µg/ml, final),
Wait 10 minutes,
Human VWF.biotin (10 µg/ml, final), followed immediately by,
Human platelet rich plasma (1 x 10^7/ml, final)

mice
① tumor cells
② α-TAA-biotin
③ avidin
④ VWF biotin
⑤ plasma

After 1 hour, the animals were euthanized (CO_2 gas) and the *following conducted in a blinded fashion* by a veterinary pathologist:

Rapid post mortem,
Tumor excised, divided into three (1 piece fixed in formalin, another fixed in glutaraldehyde and the last frozen at –70° C)
Tissue samples taken from brain, lung, liver, kidney, spleen, heart for histological analysis.

Therapy Effect on Tumor Vasculature -

Histological analysis of the tumor and normal tissues (H&E staining) were conducted in *blind fashion.* The tumor tissues were analyzed and randomly assessed by identification number. The tumor tissues were scored for degree of thrombosis and necrosis on a scale of 0 to 4, with 0

indicating no evidence of thrombosis and/or necrosis and 4 demonstrating very high levels of thrombosis and/or necrosis. Thrombosis was further broken into central thrombosis and peripheral thrombosis. One animal from both the Control and Treatment groups was excluded from analysis due to difficulties in sectioning, staining and interpreting the results (#5 and #40, respectively).

Microscopic examination of tumor tissue sections taken from the treatment group revealed extensive thrombosis of peripheral tumor vessels in comparison to control animals (i.e. animals receiving all treatment agents, with the substitution of an irrelevant, biotinylated antibody for the targeting antibody). Histological examination of brain, lung, liver, kidney, spleen and pancreas in the treatment animals showed no evidence of thrombosis.

Table 1. Thrombosis/Necrosis Data Summary

Mouse ID #	THROMBOSIS		NECROSIS
	Peripheral	Central	
3 – Control	1	4	0
4 – Control	2	2	1
11 – Control	1	3	4
15 – Control	0	0	0
16 – Control	0	2	2
20 – Control	1	4	1
24 – Control	1	1	1
25 – Control	0	1	0
27 – Control	3	4	2
	Mean = 1.0 ± 1.0	**Mean = 2.3 ± 1.5**	**Mean = 1.2 ± 1.3**
1 – Treatment	2	2	4
2 – Treatment	4	1	1
14 – Treatment	1	3	0
22 – Treatment	4	4	0
28 – Treatment	3	3	0
30 – Treatment	4	4	0
33 – Treatment	2	2	3
35 – Treatment	2	2	2
36 – Treatment	3	4	3
39 – Treatment	3	4	2
	Mean = 2.8 ± 1.0	**Mean = 2.9 ± 1.1**	**Mean = 1.5 ± 1.5**

Statistical analysis of the data in Table 1 indicate a significant difference between the degree of peripheral thrombosis in the Control and the Treatment groups ($p < 0.001$). Central thrombosis was a common feature between the two groups, as shown in Figure 9 (the difference between the two groups was not statistically significant for either central thrombosis or necrosis. However, tumor cell necrosis was noted in the vicinity of occluded peripheral tumor vasculature in the test animals.

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

November 2, 2001

M.W. Stewart

Michael W. Stewart
Inventor